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Pathogenesis of Avian Influenza A (H5N1) Viruses in Ferrets

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Highly pathogenic avian influenza A H5N1 viruses caused outbreaks of disease in domestic poultry and humans in Hong Kong in 1997. Direct transmission of the H5N1 viruses from birds to humans resulted in 18 documented cases of respiratory illness, including six deaths. Here we evaluated two of the avian H5N1 viruses isolated from humans for their ability to replicate and cause disease in outbred ferrets. A/Hong Kong/483/97 virus was isolated from a fatal case and was highly pathogenic in the BALB/c mouse model, whereas A/Hong Kong/486/97 virus was isolated from a case with mild illness and exhibited a low-pathogenicity phenotype in mice. Ferrets infected intranasally with 10^7 50% egg infectious doses (EID_{50}) of either H5N1 virus exhibited severe lethargy, fever, weight loss, transient lymphopenia, and replication in the upper and lower respiratory tract, as well as multiple systemic organs, including the brain. Gastrointestinal symptoms were seen in some animals. In contrast, weight loss and severe lethargy were not noted in ferrets infected with 10^7 EID_{50} of two recent human H3N2 viruses, although these viruses were also isolated from the brains, but not other extrapulmonary organs, of infected animals. The results demonstrate that both H5N1 viruses were highly virulent in the outbred ferret model, unlike the differential pathogenicity documented in inbred BALB/c mice. We propose the ferret as an alternative model system for the study of these highly pathogenic avian viruses.

In Hong Kong, during 1997, avian influenza A H5N1 viruses caused outbreaks of disease in domestic poultry markets. Poultry-to-human transmission of these viruses resulted in 18 documented human illnesses, including six deaths (3, 4, 42). Molecular analyses of the H5N1 viruses isolated from humans determined that all eight genes were derived from avian viruses (3, 41, 42). The viruses isolated from humans, like those isolated from poultry, were highly pathogenic for experimentally infected chickens (9, 38, 41) and possessed a series of multiple basic amino acids adjacent to the hemagglutinin cleavage site characteristic of highly pathogenic avian viruses (36). In humans, the viruses resulted in a range of clinical outcomes, from mild infections, primarily in persons <12 years of age, to severe respiratory illness and death, primarily in persons aged >12 years old (49). Complications in severe cases included acute respiratory distress syndrome, leukopenia, lymphopenia, hemophagocytosis, and multiorgan dysfunction (49). The unusual prominence of gastrointestinal symptoms, hematologic disorders, and liver and renal dysfunction in cases suggested that the H5N1 viruses had a wider tissue tropism than human influenza A H1N1 and H3N2 viruses. However, virus replication outside the respiratory tract was not demonstrated with the limited autopsy tissues that were available.

A number of laboratories, including ours, have investigated a murine model for the study of avian H5N1 virus pathogenicity in mammals (6, 9, 10, 19, 26). The H5N1 viruses grew efficiently in the respiratory tract of BALB/c mice without the prior adaptation that is usually required for human influenza A viruses to replicate in this host (39). The H5N1 viruses isolated from humans showed two distinct phenotypes in the inbred mouse. Replication of viruses of low pathogenicity was restricted to the respiratory tract, was generally nonlethal, and animals cleared the virus 7 to 9 days postinfection (p.i.). In contrast, viruses of high pathogenicity replicated in multiple systemic organs in addition to the respiratory tract and caused lymphocyte depletion and death of the animals by 6 to 9 days p.i. (9, 19, 45). While the inbred mouse model reproduced several clinical features of the human disease and the pathogenicity phenotype in mice correlated more often than not with the severity of disease in humans (15), it was important to determine whether the pathogenicity of the H5N1 viruses was similar in other, outbred mammalian hosts.

Various species have been used as nonhuman primate models for influenza (31). Squirrel monkeys (*Saimiri sciureus*) developed upper respiratory tract, but not febrile, illness after transtracheal infection with three human influenza A viruses, whereas 10 serologically distinct avian viruses showed a spectrum of upper respiratory tract replication and clinical illness (24, 25). Recently, Rimmelzwaan et al. (33) reported that A/Hong Kong/156/97, the H5N1 virus isolated from the first human case caused severe respiratory disease in cynomolgus macaques (*Macaque fascicularis*). Although this primate may offer a useful model for H5N1 pathogenesis studies, availability, cost, and ethical constraints may limit the utility of nonhuman primates in general for such research. Because ferrets (*Mustela putorius furo*) are naturally susceptible to infection with human influenza A and B viruses and their disease resembles that of human influenza, these animals have been widely used as a model for influenza virus pathogenesis and immunity studies (1, 7, 14, 48; for reviews, see references 40

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and 43). The relative virulence of certain influenza A viruses was shown to be similar in humans and ferrets (2, 21, 44). Ferrets are also susceptible to avian, equine, and swine influenza A viruses, although only human and swine viruses were reported to induce febrile illness (13, 22, 37).

Here we evaluate the ferret as a model for the study of avian influenza virus H5N1 pathogenesis in mammals. Two H5N1 viruses were compared for their ability to replicate and cause disease in outbred ferrets. A/Hong Kong/483/97 (HK/483) virus was isolated from a fatal case in a 13-year-old patient and was highly pathogenic in the BALB/c mouse model (9, 19, 45). A/Hong Kong/486/97 virus was isolated from a 4-year-old child with mild illness and exhibited a low-pathogenicity phenotype in mice. Our results indicate that the ferret may be considered as a reasonable alternative for the study of these highly pathogenic avian viruses in an outbred mammalian system.

MATERIALS AND METHODS

Viruses. The avian H5N1 viruses A/Hong Kong/486/97 (HK/486) and A/Hong Kong/483/97 (HK/483) and the human H3N2 viruses A/Sydney/05/97 (Sydney/97) and A/Panama/2007/99 (Panama/99) were used in this study. A U.S. Department of Agriculture permit was obtained for work with avian influenza viruses. H5N1 virus stocks were grown in MDCK cells either once (HK/483) or twice (HK/486) and were then grown in the allantoic cavities of 10-day-old embryonated hens' eggs for 24 to 28 h at 37°C for four passages. H5N1 viruses were handled under biosafety level 3+ (BSL-3+) laboratory conditions (34, 51).

H3N2 viruses were grown in embryonated eggs for 48 h at 34°C; Sydney/97 and Panama/99 were passaged three and five times, respectively, in our laboratory. Virus stocks were aliquoted and stored at −70°C until use. Fifty percent egg infectious dose (EID_{50}) titers were calculated by the method of Reed and Muench (30) after serial titration in eggs.

Infection and monitoring of ferrets. Young adult male or female ferrets (Marshall Farms, North Rose, N.Y.) aged 8 to 10 months and serologically negative by hemagglutination inhibition assay for currently circulating human influenza A or B viruses were moved at least 4 days prior to infection to the BSL-3+ animal holding area, where they were housed in cages contained in bioclean portable laminar flow clean room enclosures (Lab Products, Seaford, Del.). Prior to infection, baseline temperatures were measured twice daily for at least 3 days. Ferrets were anesthetized with ketamine (25 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg) by the intramuscular route and infected intranasally (i.n.) with a total of 1 ml of 10^7 EID_{50} of virus/ml in phosphate-buffered saline (PBS) delivered to the nostrils. For both H5N1 viruses, this dose was approximately equivalent to 10^5 50% ferret infectious doses (FID_{50}). Control animals were mock infected with an equivalent dilution (1:30) of noninfectious allantoic fluid. Temperatures were measured twice daily using either a rectal thermometer or a subcutaneous implantable temperature transponder (BioMedic Data Systems, Inc., Seaford, Del.). Preinfection values were averaged to obtain a baseline temperature for each ferret. The change in temperature (in degrees Celsius) was calculated at each time point for each animal. Clinical signs of sneezing (before anesthesia), inappetence, dyspnea, and level of activity were assessed daily. A scoring system based on that described by Reuman et al. (32) was used to assess the activity level as follows: 0, alert and playful; 1, alert but playful only when stimulated; 2, alert but not playful when stimulated; and 3, neither alert nor playful when stimulated. Based on the daily scores for each animal in a group, a relative inactivity index was calculated as follows: $\Sigma_{(\text{day 1 to day 7})}\,[\text{score} + 1]_n/\Sigma_{(\text{day 1 to day 7})}\,n$, where n equals the total number of observations. A value of 1 was added to each base score so that a score of 0 could be divided by a denominator, resulting in an index value of 1.0. The numbers of animals assessed on different days were as follows: days 0 and 1, $n = 9$; day 3, $n = 7$; day 5, $n = 5$; and days 7 and 9, $n = 3$.

The FID_{50} was determined for each virus by i.n. infection of two ferrets each with doses of 10^4, 10^3, and 10^2 EID_{50} of virus and three ferrets each with 10^1 EID_{50} of virus as described above. Nasal wash samples were collected on day 3 p.i. and titrated in eggs to detect the infectious virus. Animals with nasal wash titers of $\geq 10^2$ EID_{50}/ml were considered positive for virus. The FID_{50} was calculated by using the method of Reed and Muench (30).

Collection of nasal wash, blood, and tissue samples. Nasal washes were collected 4 to 6 h after inoculation and on days 1, 3, 5, 7, 9, and 11. Ferrets were sedated with ketamine (25 mg/kg), and 0.5 ml of sterile PBS containing 1% bovine serum albumin and penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) was injected into each nostril and collected in a petri dish when expelled by the ferret. The volume was brought up to 1 ml with cold sterile PBS plus antibiotics. Sedated ferrets were weighed and bled via venipuncture of the anterior vena cava on days 1, 3, 5, 7, 9, 11, and 14 p.i. Then, 1 ml of blood was collected in heparanized tubes. Differential blood counts were performed on all animals in each group. Fecal swabs were collected and stored in 1 ml of cold sterile PBS containing antibiotics as indicated above. All tissue samples, nasal washes, and fecal swabs were immediately placed on dry ice and subsequently stored at −70°C until further analyses. On days 1, 3, 5, 7, and 14, two to three animals were euthanized by intracardiac injection of Euthanasia V solution (1 ml/kg of body weight). Tissues from the nasal turbinates and all major organs, including the brain, were collected and either frozen on dry ice for virus isolation or placed in formalin for histologic analyses.

Virus titrations. Frozen ferret tissues were thawed, weighed, and homogenized by using a mortar and pestle to which a small volume of sterile glass beads (2 mm in diameter) and sterile, cold PBS plus antibiotics was added to facilitate homogenization. Solid debris was pelleted by centrifugation, and tissues were titrated for virus infectivity in eggs. Virus titers are expressed as EID_{50}/gram for solid tissues and EID_{50}/milliliter for nasal washes and turbinates. The limits of virus detection were $10^{1.0}$ EID_{50}/ml for nasal wash or turbinate and $10^{1.0}$ EID_{50}/g for solid organs.

Histopathologic and immunohistochemical analysis. Two or three ferrets were euthanized on days 1, 3, 5, and 14 p.i. and tissues were removed and fixed in formalin, routinely processed, and embedded in paraffin. Routine hematoxylin-and-eosin (H&E)-stained sections were examined. For antigen staining, sections were processed for immunohistochemistry by using a two-step biotin-streptavidin method essentially as described previously (50) and a monoclonal antibody to influenza A nucleoprotein as the primary antibody.

Statistical significance. Differences in weight and virus titer data were tested for significance by using Student's *t* test. Differences in temperature over the time course of infection were analyzed by using general linear modeling in SAS (SAS/STAT software changes and enhancements, version 8; SAS Institute, Inc., Cary, N.C.). This model takes into account the relative change in temperature over time and differences in animal numbers at different time points of infection.

RESULTS

Clinical signs of ferrets infected with A/HK/483/97 and A/HK/486/97 viruses. Nine ferrets were inoculated i.n. with 10^7 EID_{50} ($10^5 FID_{50}$) of either HK/483 or HK/486 virus. This dose has been reported to consistently infect ferrets with human (40) or avian (13) influenza viruses. Animals were monitored for clinical symptoms and outcome for 14 days. Two animals from each group were euthanatized on days 1, 3, and 5 p.i. for the collection of tissues. By day 3 p.i., there was a substantial decrease in activity in most animals infected with either virus (Table 1). By day 5 p.i., all animals infected with HK/486 were neither playful nor alert and this extreme lethargy continued into the second week after infection. Likewise, animals infected with HK/483 were lethargic up to day 8 p.i., regaining some level of activity thereafter. The relative inactivity index was 1.9 for HK/483-infected ferrets and was 2.6 for HK/486-infected animals, which reflected the more severe lethargy observed in the latter group. Ferrets infected with either virus exhibited clinical signs of respiratory disease, including nasal discharge, sneezing, and visual signs of dyspnea. Investigators were required to wear purified air-powered respirators that precluded aural evaluation of rales and wheezing. Ferrets infected with either virus developed yellow-colored diarrhea that began earlier in ferrets infected with HK/483 virus. Ferrets infected with either H5N1 virus exhibited substantial weight loss over the course of infection (Fig. 1B). Both H5N1 viruses induced a transient lymphopenia in ferrets, with a loss of 60 to 65% of peripheral blood lymphocytes by day 3 p.i (data not

TABLE 1. Clinical signs following infection of ferrets with HK/483 or HK/486 H5N1 viruses

Day p.i.	*n*	No. of ferrets with clinical signs															
		Decreased activity[a]		Inappetence[b]		Nasal discharge[c]		Sneezing		Dyspnea[d]		Diarrhea		Conjunctival discharge[e]		Neurologic signs[f]	
		483	486	483	486	483	486	483	486	483	486	483	486	483	486	483	486
1	9	–[g]	–	1	–	4	–	–	–	–	–	1	–	–	–	–	–
2	7	5	3	–	–	3	2	–	1	–	–	2	–	1	–	–	–
3	7	7	6	2	1	3	2	1	–	–	–	3	–	–	–	–	–
4	5	5	5	–	–	2	1	–	3	1	–	3	1	–	–	–	–
5	5	4	5	2	–	3	2	1	–	–	–	–	1	1	–	–	–
6	3	2	3	–	–	1	–	–	–	–	–	–	–	–	–	–	–
7	3	2	3	–	–	–	–	2	–	–	–	–	–	–	–	–	–
8	3	2	3	–	–	1	–	–	–	–	–	1	–	–	–	–	–
9	3	–	3	–	–	–	–	–	–	–	1	–	1	–	–	–	–
10	3	–	2	–	3	–	–	–	–	–	–	–	–	–	1	–	1
11	3	–	3	–	2	–	1	–	1	–	–	–	–	–	1	–	2
12	3	–	3	–	3	–	1	–	–	–	–	–	–	–	1	–	2
13	3	–	3	–	3	–	1	–	–	–	–	–	–	–	1	–	2
14	3	–	3	–	3	–	1	–	–	–	–	–	–	–	1	–	2

[a] Number of animals with activity scores of ≥1.0 determined as described in Materials and Methods.
[b] Decreased appetite was based on absence of food in the stomach at necropsy or failure to remove food from the food hopper.
[c] HK/483-infected ferrets developed a serous nasal discharge, while HK/486-infected ferrets developed a mucopurulent nasal discharge.
[d] Ferrets exhibited open-mouth breathing with exaggerated abdominal movement. Aural assessment by investigator was not possible due to the use of a personal air-powered respirator.
[e] HK/483-infected ferrets developed a serous ocular discharge, while HK/486-infected ferrets developed a mucopurulent ocular discharge.
[f] Neurological signs were hind-limb paresis, ataxia and torticollis. In another experiment, two ferrets per group were infected with 10^4, 10^3, or 10^2 EID_{50} (equal to 100, 10, and 1 FID_{50}, respectively) of HK/483 or HK/486. On day 7 p.i., one of the two ferrets infected with each dose of HK/483 exhibited the aforementioned neurological signs. One of two ferrets infected with 10^3 or 10^2 EID_{50} of HK/486 neurological signs on days 8 and 13 p.i., respectively. In a third experiment, three of four ferrets infected i.n. with 10^7 EID_{50} (10^5 FID_{50}) of HK/486 exhibited neurological signs.
[g] –, No animals had a particular clinical sign.

shown). Lymphocyte numbers remained depleted on day 5 p.i. but had recovered to, or were approaching, preinfection levels by day 7 p.i. Ferrets infected with HK/486 virus developed neurological symptoms, including ataxia, hind-limb paresis, and torticollis, beginning on day 10 p.i. One ferret infected with HK/486 virus died on day 9 p.i. The occurrence of neurological symptoms was observed repeatedly in H5N1-infected animals. In other experiments, three of four animals infected with 10^7 EID_{50} of HK/486 and two of six ferrets and three of six ferrets infected with lower doses of HK/486 and HK/483, respectively, also developed hind-limb paresis and torticollis 7 to 13 days after infection and were euthanatized (see Table 1, footnote *f*).

Kinetics of virus replication in the upper respiratory tract and weight loss. Figure 1 presents the kinetics of virus replication in the upper respiratory tract and morbidity, as measured by weight loss, in ferrets infected with HK/483 or HK/486. The mean peak viral titer in ferrets infected with HK/486 virus ($10^{5.0}$ EID_{50}/ml) was detected as early as day 1 p.i., while a similar peak mean titer was detected in HK/483-infected ferrets ($10^{4.8}$ EID_{50}/ml) on day 3 p.i. (Fig. 1A). Virus titers in HK/483- and HK/486-infected animals declined to levels below detection by days 7 and 9 p.i., respectively (Fig. 1A). The kinetics of upper respiratory tract replication of the two H5N1 viruses were similar to those observed for recent human H3N2 viruses, although peak nasal wash titers of the human H3N2 viruses were at least 100-fold higher than those detected in H5N1-infected animals (data not shown). Ferrets infected with either H5N1 virus began to lose weight as early as 1 day after infection (Fig. 1B) and continued to lose weight as the infection progressed. By day 11 p.i., the mean percent weight loss in ferrets infected with HK/483 was 29%, while ferrets infected with HK/486 had a mean weight loss of 15% ($P = 0.002$). The weights of both groups of ferrets increased after this time.

Change in temperatures of ferrets infected with H5N1 viruses. Temperatures of individual ferrets were monitored in the morning and evening for 3 days prior to infection and for 14 days after infection. Figure 2 presents the mean change in temperature from baseline after infection of animals with either HK/483 or HK/486 virus. Temperatures through day 9 p.i. only are shown since temperatures had returned to baseline levels by this time. Ferrets infected with either virus developed a significant increase (>3 SD above the baseline values) in body temperatures by day 1 p.i and a peak mean temperature rise of 1.8°C on day 2 p.i. The mean temperature of HK/483-infected ferrets was above baseline from days 1 to 4 and days 5 to 8 p.i., whereas the mean temperature of HK/486-infected ferrets remained above baseline for 8 days p.i. Using a general linear model analysis, the rise in temperature over the entire time period monitored (−3 to 14 days p.i.) was significantly greater for HK/486- compared with HK/483-infected animals ($P = 0.0002$).

Replication of H5N1 avian influenza viruses in respiratory tract and other tissues. We next investigated the ability of the H5N1 viruses to infect the lower (lungs) compared with the upper (nasal turbinates) respiratory tract and to spread to organs outside of the respiratory tract. Figure 3 presents the viral titers in different organs from individual animals. Both HK/483 and HK/486 replicated with similar kinetics in nasal turbinates in which virus persisted at high titers for at least 5 days p.i. In one experiment, HK/483 virus was recovered from nasal turbinates at low titers (mean titer, $10^{2.6}$ EID_{50}/g) on day 7 p.i. and was not detectable by day 9 p.i. (data not shown).

Both viruses replicated to substantial titers in the lungs of ferrets, although virus was not isolated from the lungs of all animals at all time points tested. Peak virus titers were isolated on day 1 p.i. from lungs of HK/483-infected ferrets and from HK/486-infected ferrets on day 3 p.i. Although the mean lung virus titer on day 3 p.i. was 1,000-fold higher in HK/486-infected ferrets (mean ± the standard error [SE], $\log_{10}$ 5.2±1.6 EID_{50}/g) compared with HK/483-infected ferrets (mean ± SE, $\log_{10}$ 2.2 ± 0.6 EID_{50}/g), this difference was not statistically significant due to the variability in individual titers. Virus was also isolated from the brains of HK/483-infected ferrets on day 3 p.i and from some HK/486-infected ferrets on days 1, 3, or 5 p.i. Likewise, virus was isolated from the spleens and intestines of animals infected with either H5N1 virus. HK/483 virus was detected in spleen on days 3 and 5 p.i. and in the intestine on days 1, 3, and 5 p.i. Detection of HK/486 virus in these tissues was somewhat more limited. HK/486 was detected in the spleen only on day 3 and in the intestines of some animals primarily on days 1 and 3 p.i. Low titers of virus ($10^{2.0}$ EID_{50}/ml) were recovered from fecal swabs collected from 1 of 9 HK/483-infected ferrets on day 1 p.i. and 2 of 5 ferrets on day 5 p.i. (data not shown). No virus was detected in fecal swabs from HK/483-infected ferrets after day 5 p.i. or at any time point from animals infected with HK/486. Virus was detected on day 5 p.i. in the liver of one animal infected with HK/483 ($10^{2.5}$ EID_{50}/g). Low titers of virus were isolated on day 3 p.i. from the hearts of HK/486-infected ferrets (mean titer = $10^{1.7}$ EID_{50}/g) and on day 14 p.i. from the liver of one animal ($10^{1.5}$ EID_{50}/g). With the latter exception, no virus was isolated from any other tissues collected on day 14 p.i. from ferrets infected with either H5N1 virus. Taken together, these results indicate that both HK/483 and HK/486 replicated extensively in both the upper and the lower respiratory tract of ferrets and were also isolated from multiple systemic organs.

Gross and histologic lesions in H5N1-infected ferrets. Macroscopic lesions were observed most frequently in the lungs of H5N1-infected animals. Focal areas of redness were present in the lungs of HK/486-infected ferrets by as early as day 1 p.i. and in HK/483-infected animals by as early as day 3 p.i. Consolidation of the lungs was evident in ferrets infected with either virus by day 3 p.i. Lung lesions were more diffuse on days 5 to 14 p.i. Other extrapulmonary lesions that were seen less frequently in ferrets infected with either H5N1 virus on days 3 or 5 p.i. included both discoloration of or petechiae on the liver and lesions on the intestines and kidneys.

A spectrum of histopathologic features were found in the lungs and brains of ferrets infected with either HK/483 or HK/486. Representative microscopic findings, primarily from HK/486-infected ferrets, are presented in Fig. 4. Acute bronchiolitis, bronchopneumonia, and interstitial pneumonia were observed in the lungs of ferrets infected with HK/486 as early as day 1 p.i and in HK/483-infected ferrets by day 3 p.i. Severe bronchopneumonia observed on day 3 in a HK/486-infected ferret (Fig. 4A) was characterized by suppurative exudates in the bronchi, bronchioles, and adjacent alveolar spaces. Prominent epithelial necrosis and marked intraalveolar edema were also present. In comparison, the lung from a mock-infected control ferret had no apparent histologic changes (Fig. 4C), and the lung of a ferret infected with 10^7 EID_{50} of the H3N2 virus Panama/99 showed interstitial pneumonia on day 3 p.i.

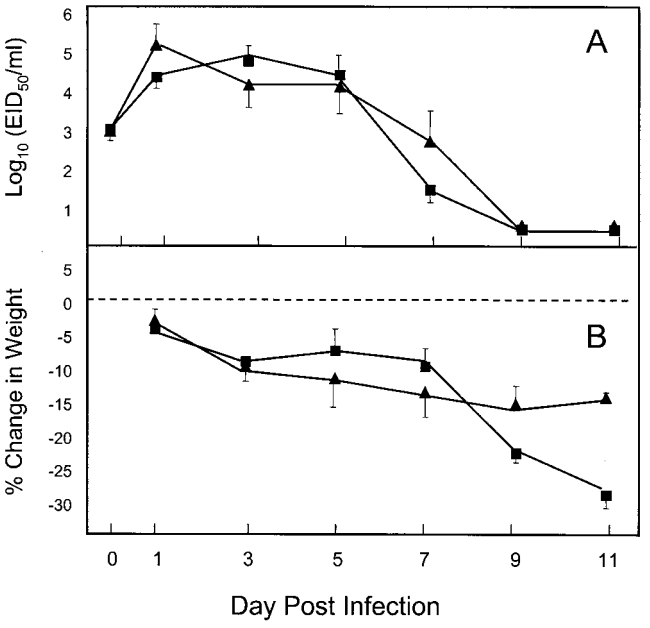

FIG. 1. Kinetics of virus replication in the upper respiratory tract and weight loss in H5N1-infected ferrets. Nine ferrets were inoculated i.n. with 10^7 EID_{50} of either HK/483 (■) or HK/486 (▲). The number of animals assessed on different days was as follows: days 0 and 1, n = 9; day 3, n = 7; day 5, n = 5; and days 7, 9 and 11, n = 3. (A) Nasal wash samples were collected from ferrets on the days indicated; virus titers were determined in eggs and are expressed as the $\log_{10}$ mean ± SE EID_{50}/ml. The limit of virus detection was $\leq 10^{1.0}$/ml. (B) Weights of animals were recorded on the days indicated, and the individual differences in weight compared with weights prior to infection were calculated for each individual animal. The mean percent change in weight is presented. Weight loss in HK/483-infected ferrets was greater than in HK/486-infected ferrets on day 11 p.i. (P = 0.002). The change in weight in six mock-infected control animals ranged from −1.4 to +3.9%.

(Fig. 4D) and bronchiolitis (data not shown). Interstitial inflammatory infiltrate was still evident in the lungs of H5N1-infected ferrets euthanatized on day 14 p.i. (data not shown). Immunohistochemical staining for the presence of viral antigen yielded rare positive cells in the lungs of H5N1-infected animals (Fig. 4B). Histopathologic features detected in the brains of ferrets infected with either H5N1 virus from days 5 onward included the presence of glial nodules (Fig. 4E and F), perivascular infiltration of lymphocytes and polymorphonuclear cells in the parenchyma (Fig. 4G), neuronophagia (Fig. 4H), and increased lymphocytic infiltrate in the choroid plexus (data not shown). Although the spectrum of histopathologic features in the lungs and brains were similar in ferrets infected with either H5N1 virus, the lesions seen in HK/483-infected animals were, in general, less severe and/or appeared later in infection. The extensive histologic changes in the brains of HK/486-infected animals were consistent with neurological symptoms observed in some animals late in the disease process.

Comparison of clinical symptoms and virus isolation among ferrets infected with 10^7 EID_{50} of H5N1 or H3N2 viruses. To assess the relative virulence of the avian H5N1 viruses in ferrets, two additional groups of ferrets were infected with 10^7 EID_{50} of the recent human H3N2 viruses Sydney/97 (n = 5) or

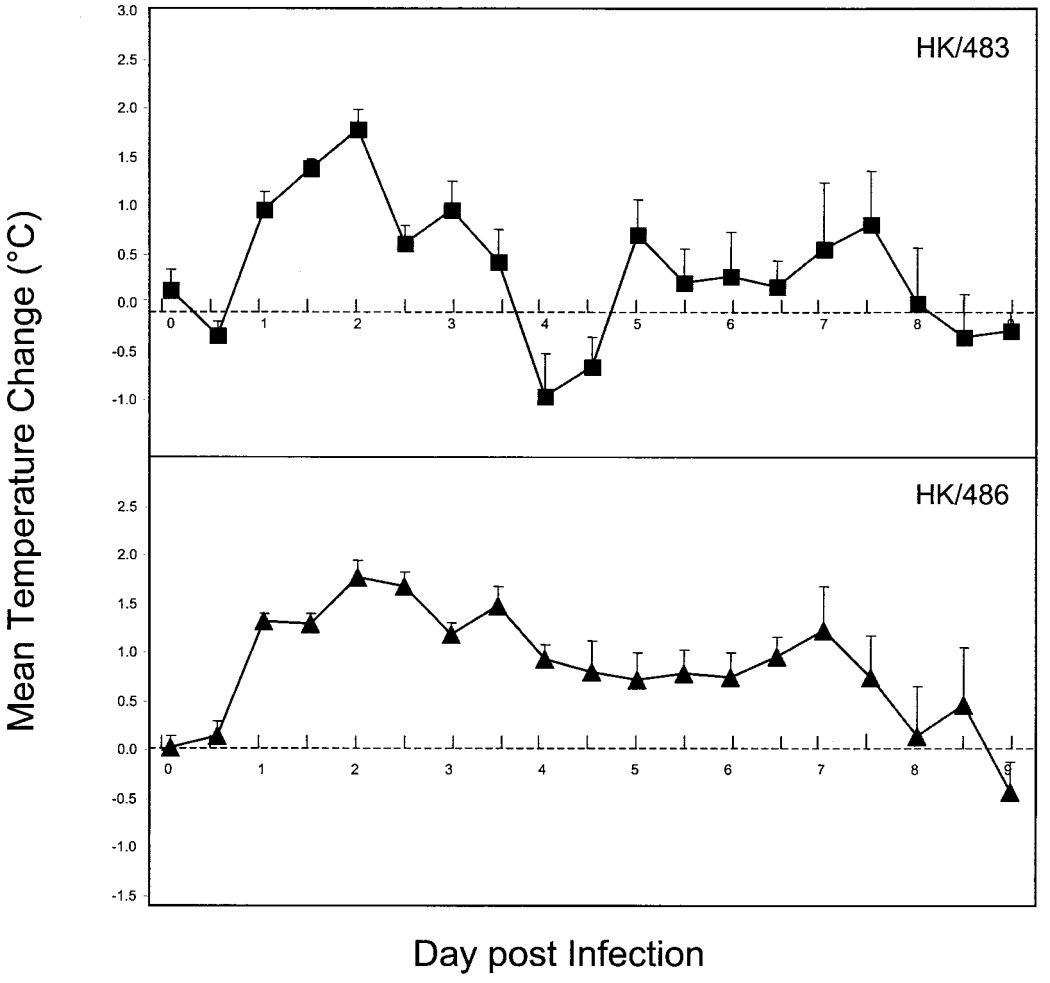

FIG. 2. Change in temperatures of ferrets infected with H5N1 viruses. Ferrets were infected with 10^7 EID_{50} of either HK/483 or HK/486. Temperatures were assessed twice daily, beginning 3 days prior to infection and for 14 days after infection. The numbers of animals monitored each day were as follows: days −3 to 1, $n = 9$; days 2 and 3, $n = 7$; days 4 and 5, $n = 5$; and days 7 to 14, $n = 3$. The means of preinfection temperatures (days −3 to −1) were subtracted from the individual p.i. temperatures to obtain temperature changes in individual ferrets. The mean change in temperature for all ferrets in a group is shown. Three ferrets inoculated with each virus were sampled on remaining days. The mean temperature change in six mock-infected control ferrets ranged from −0.5 to +0.4°C.

Panama/99 ($n = 4$). Table 2 compares virulence parameters for H5N1-infected and H3N2-infected animals, including the FID_{50}, activity level, weight loss, fever, and extent of viral replication in the upper and lower respiratory tract or in systemic organs over the first 7 days of infection. The FID_{50} of the H5N1 viruses were similar to each other but were 5- to >20-fold higher than those of the H3N2 viruses, indicating that fewer infectious virus particles of H3N2 virus were needed to infect the animals. Viral titers in nasal turbinates in H5N1-infected ferrets on day 3 p.i. were at least 1,000-fold lower than those detected in human H3N2-infected animals. Nevertheless, H5N1-infected ferrets had higher mean peak temperature rises, and scored substantially higher by using the relative inactivity index (1.9 to 2.6) compared with H3N2-infected animals (1.0 to 1.2). This latter difference in the severity of clinical illness was exemplified by the dramatic and significant weight loss observed in H5N1-infected ferrets compared with a modest weight gain detected in H3N2-infected animals ($P \leq 0.01$). While both H5N1- and H3N2-infected ferrets exhibited transient lymphopenia, the degree of lymphocyte depletion was significantly greater in H5N1-infected animals ($P < 0.001$). While both H5N1 viruses were isolated from lungs on day 3 p.i., infection with only one of the H3N2 viruses (Panama/99) yielded virus from the lungs on day 3 p.i. Histopathologic features found in the lungs of H3N2-infected animals included bronchiolitis and interstitial pneumonia but were substantially less severe than those detected in H5N1-infected animals (data not shown). To our surprise, virus was isolated from the brains of ferrets infected with either H3N2 virus, as well as from the H5N1-infected animals, as presented earlier. However, none of the H3N2-infected ferrets showed any neurological signs. While virus was isolated from the spleen and intestine of H5N1-infected ferrets, no virus was detected in these organs from H3N2-infected animals. Taken together, these results suggest that infection of ferrets with the H5N1 viruses caused substantially more severe clinical disease, despite the less-efficient replication of these viruses in the upper respiratory tract compared to H3N2 viruses.

DISCUSSION

Infection of ferrets with two highly pathogenic avian H5N1 influenza viruses resulted in upper and lower respiratory tract infection, severe lethargy, fever, weight loss, transient lymphopenia, and gastrointestinal symptoms (diarrhea) in some animals. Both H5N1 viruses were isolated from multiple systemic organs, albeit at substantially lower titers compared with those from respiratory tract tissues. Histopathological features of the H5N1 infections were consistent with broncho- and interstitial pneumonia. Compared with two recent human H3N2 viruses, the H5N1 viruses induced a respiratory disease of substantially greater severity, despite the fact that the H3N2 viruses replicated 100 to 1,000 times more efficiently in the upper respiratory tract of ferrets. These results suggest that the ferret may be a useful model for the further study of avian H5N1 virus pathogenesis in mammals.

This is the first demonstration of a nonadapted avian influenza virus causing severe disease in ferrets. Previous studies reported either a subclinical infection with a range of avian virus subtypes (13) or a mild rhinitis in the absence of fever after infection with A/Turkey/Ontario/7732/66 (H5N9) virus, which is highly virulent in turkeys and chickens (22). Likewise, an avian H7N7 virus isolated from seals replicated but failed to cause disease in ferrets (35, 47). However, a laboratory-selected variant of the H7N7 virus that had acquired a multibasic cleavage site in HA (18) exhibited greater virulence for ferrets, including replication in extrapulmonary tissues, and with further passage in ferrets was highly lethal for this host (35). Furthermore, the avian H5N1 viruses which have been shown to preferentially bind sialic acid (SA) α2,3-galactose structures (23) readily replicated in the lungs of ferrets. A previous study reported that a SA α2,3-galactose-binding variant of human H3N2 virus replicated less well in ferret lungs and caused less-severe clinical signs compared with the SA α2,6-galactose-binding wild-type parental virus (16).

Although there was no direct evidence of viral spread outside of the respiratory tract in human H5N1 cases, multiorgan dysfunction was associated with severe disease (49). Some of the deaths in H5N1 cases occurred late in the course of hospitalization after extensive periods of mechanical ventilation. In the present study only one ferret died as a result of H5N1 infection, although several animals were euthanatized because of neurological signs. Ferrets infected with HK/486 demonstrated neurological symptoms of torticollis, ataxia, and hindlimb paresis beginning on day 10 p.i. Similar neurological symptoms were observed in chickens and mice experimentally infected with highly pathogenic avian H5 viruses and were associated with virus replication in the central nervous system (CNS) (19, 27, 45). Neurological symptoms were not a feature of human H5N1 infections. However, influenza virus-associated encephalitis has been reported as a rare complication of human influenza virus infection. Although fragments of influenza A viral genome have been amplified from the CNS of some such cases, active virus replication in the CNS has not been demonstrated (8). Interestingly, Rimmelzwaan et al. (33) demonstrated transient expression of viral M gene RNA by reverse transcription-PCR in the brains of two of four cynomolgus macaques infected with A/Hong Kong/156/97 (H5N1) isolated from the first case, although infectious virus was not isolated from brain tissues (33).

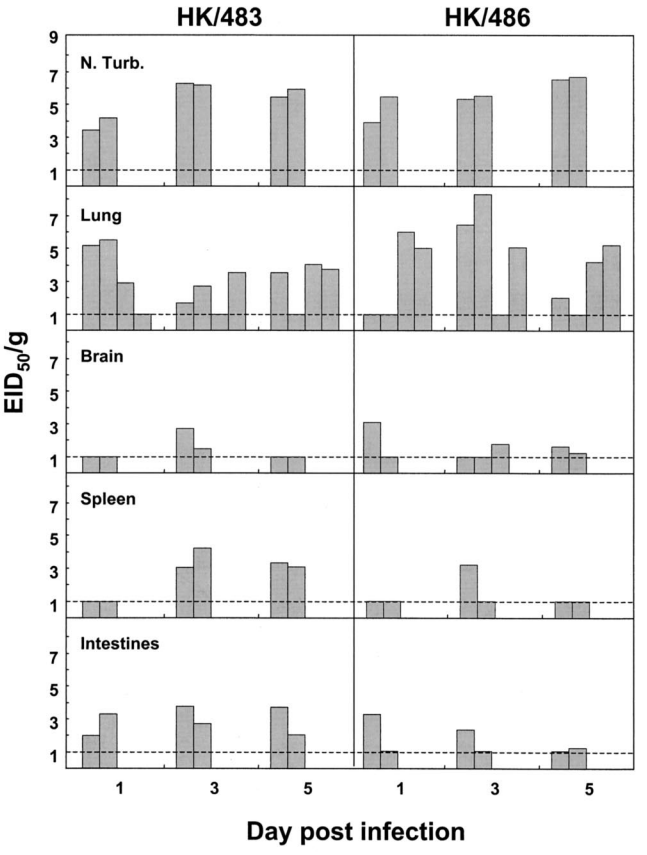

FIG. 3. Replication of H5N1 viruses in ferret tissues. Ferrets were infected with 10^7 EID_{50} of either HK/483 or HK/486. Tissues were harvested from two to four animals on the indicated days and titers were determined in eggs. For solid tissues, viral titers are expressed as $\log_{10}$ EID_{50}/g, and for nasal turbinates, titers are expressed as $\log_{10}$ EID_{50}/ml. The limit of virus detection was $\leq 10^{1.0}$/ml for nasal turbinates and $\leq 10^{1.0}$/g for all other tissues.

Smith and Sweet (40) used four markers for the determination of the relative virulence of influenza A viruses in the ferret. They were the 50% minimal infectious dose, referred to here as the FID_{50}, the extent of pyrexia and the extent of virus replication in the upper and lower respiratory tract. Two other measures of morbidity used in the present study were weight loss and activity level. In addition, the ability of avian viruses to replicate outside of the respiratory tract is considered a measure of virulence. Of these criteria, weight loss was significantly greater in HK/483-infected ferrets at a single time point (Fig. 1), whereas the extent of pyrexia over the time course of infection was significantly greater in HK/486-infected animals. Although HK/486-infected ferrets exhibited a greater qualitative difference in the degree of inactivity, no appreciable quantitative differences in the extent of replication in the respiratory tract or other organs was observed. Therefore, at the infectious dose used in this study (10^7 EID_{50} per ferret), no consistent differences in virulence between the two H5N1 viruses were established. Further studies with lower doses of virus to infect ferrets may distinguish some differences between these H5N1 viruses. However, compared to recent human

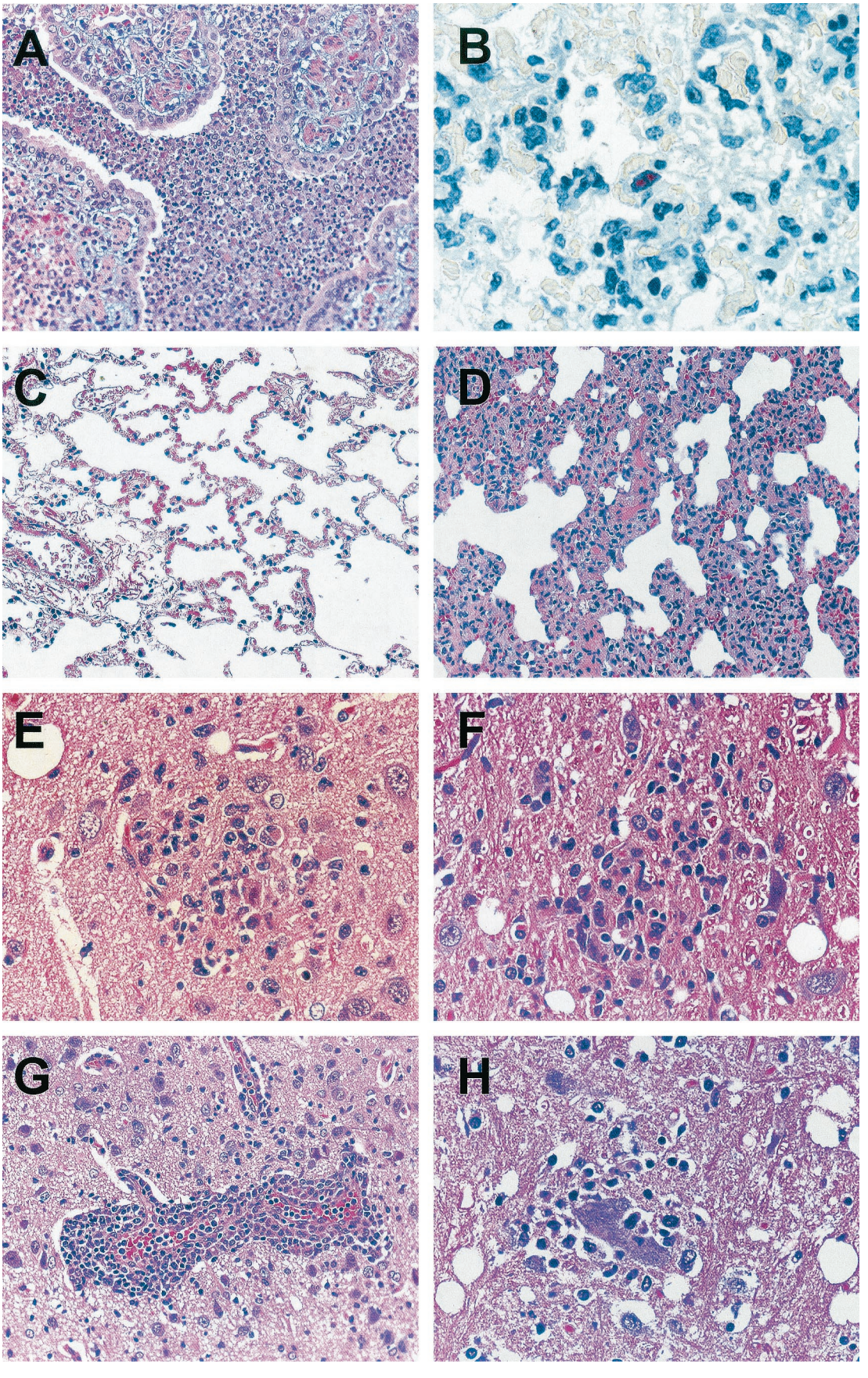
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TABLE 2. Comparison of clinical symptoms and virus isolation for first 7 days p.i. among ferrets infected with influenza A H5N1 or H3N2 viruses[a]

Virus	FID_{50} (log_{10} EID_{50})	Clinical signs				Virus titer on day 3 p.i. (log_{10} EID_{50}/g)				
		Relative inactivity index[b]	Maximal change in:							
			Wt (%)	Temp (°C)	PBL (%)	Nasal turbs[c]	Lung	Brain	Spleen	Intestine
H5N1										
HK/483	2.0	1.9	−10.0[d]	1.8	−65[e]	6.3	2.2	2.1	3.6	2.0
HK/486	2.0	2.6	−11.7[d]	1.8	−60[f]	5.4	5.2	1.3	2.0	1.5
H3N2										
Sydney/97	≤0.7	1.0	+3.9	1.1	−22	≥10.7	≤1.5[g]	2.2	≤1.0	≤1.0
Panama/99	1.3	1.2	+8.0	1.3	NT	≥8.5	2.9	1.8	≤1.0	≤1.0

[a] Ferrets were infected i.n. with 10^7 EID_{50} of the indicated H5N1 or H3N2 viruses. Groups of nine animals were infected with the H5N1 viruses and groups of four or five animals were infected with the H3N2 viruses. PBL, peripheral blood lymphocytes.
[b] Relative inactivity index was calculated from daily activity scores as described in Materials and Methods.
[c] Virus titers in nasal turbinates (turbs) are expressed as the log_{10} EID_{50}/milliliter.
[d] $P \leq 0.01$ compared with mean weight change in H3N2-infected animals on day 5 or 7 p.i.
[e] $P = 0.0004$ compared with mean decrease in percentage of PBL in H3N2-infected animals on day 1 p.i. (22%).
[f] $P = 0.0004$ compared with mean decrease in percentage of PBL in H3N2-infected animals on day 3 p.i. (14%).
[g] The limit of virus detection in H3N2-infected ferret lungs was $10^{1.5}$ EID_{50}/g.

H3N2 viruses, the two avian H5N1 viruses induced in ferrets a substantially more severe disease that was evident from the severe weight loss and inactivity of H5N1-infected animals. Surprisingly, both H3N2 viruses were isolated from the brains of infected ferrets. This latter effect may have been, in part, associated with the very high titers of virus detected in the nasal turbinates of H3N2-infected animals (Table 2). Whether H3N2 or H5N1 viruses can be isolated from brains of ferrets infected with lower virus doses is currently under investigation. Nevertheless, severe morbidity and neurological signs were observed only in H5N1-infected ferrets, even when lower doses of virus were used to infect animals.

Two features of pathogenicity of avian H5N1 viruses differed between the ferret model and the previously described BALB/c mouse model (9, 19, 45). First, HK/486 was characterized as a low-pathogenicity virus in BALB/c mice and did not spread outside of the respiratory tract, whereas the virus was isolated from multiple systemic organs in the ferret, including the brain, spleen, and intestine. Second, HK/483, characterized as a virus of high pathogenicity in mice, caused extensive and continuous depletion of peripheral blood lymphocytes until the death of mice around days 6 to 9 p.i. (45). In contrast, ferrets infected with HK/483 (or HK/486) experienced only a transient depletion of lymphocytes similar to that previously reported for ferrets infected with the laboratory-adapted strain A/Puerto Rico/8/34 (11). One reason for the apparent differences between the mouse and ferret models may be that only a small number of genetic differences between HK/483 and HK/486 are required to influence the biological properties of the viruses in a genetically homogeneous inbred mouse versus the genetically heterogeneous ferret, i.e., the molecular changes that have been correlated with differences in H5N1 virus pathogenicity in BALB/c mice (12, 15) may not have the same effect in ferrets. It is not known whether all 16 H5N1 viruses isolated from humans will behave similarly in ferrets. The evaluation of a limited number of additional H5N1 viruses is planned. While the inbred mouse may be a convenient model to further investigate the molecular basis of pathogenesis, the ferret may be useful to investigate the contribution of host-related factors of H5N1 pathogenesis in mammals.

Compared to the mouse or ferret model, a broader spectrum of disease was seen in humans in whom the age of the patient was associated with disease severity (49). In addition, other unknown host risk factors may also have contributed to the clinical outcome in humans. For example, a more vigorous immune response in some cases may have contributed to the severity of disease. The apparent lack of detection of H5N1 virus outside the respiratory tract in humans may be due to the limited number of patients studied, the inability of the virus to escape the respiratory tract, or the greater resistance of human extrapulmonary organs to H5N1 infection. Rimmelzwaan et al. recently established a primate model for studying H5N1 virus pathogenesis (33). The respiratory tract was the major target of H5N1 virus replication in cynomolgus macaques infected with A/Hong Kong/156/97. While the primate model reproduced the acute respiratory symptoms observed in humans, additional symptoms that were associated with human H5N1 infection (49), such as leukopenia or lymphopenia and gastroin-

FIG. 4. Representative histolopathologic changes and immunostaining in tissues from ferrets infected with 10^7 EID_{50} of H5N1 or H3N2 virus. Tissues were removed at the indicated times p.i. and were processed for H&E and immunohistochemical staining. (A) H&E staining of the lungs of an HK/486-infected animal on day 3 p.i. showing extensive bronchiolar inflammation, necrosis of bronchial epithelium, and suppurative exudates in the bronchiolar lumen. (B) Immunostaining of influenza virus in lung of an HK/486-infected animal on day 3 p.i. Immunoalkaline phosphate staining, naphthol fast red substrate with light hematoxylin counterstain. (C) H&E staining of lung collected on day 3 p.i. from mock-infected control ferret showing normal histology. (D) H&E staining of lung of H3N2 (Panama/99)-infected ferret on day 3 p.i. showing interstitial pneumonitis. (E) H&E staining of brain of an HK/486-infected animal on day 5 p.i. showing presence of glial nodules. (F) H&E staining of brain of an HK/483-infected animal on day 14 p.i. showing presence of glial nodules. (G) H&E staining of brain of an HK/486-infected animal on day 14 p.i. showing prominent perivascular infiltrate. (H) H&E staining of brain of an HK/486-infected animal that died on day 9 p.i. showing neuronophagia. Magnifications: ×48 (A), ×48 (B), ×48 (C), ×48 (D), ×96 (E), ×96 (F), ×48 (G), ×96 (H).

testinal involvement, were not reported (33). Gastrointestinal involvement was a feature of H5N1 infection in ferrets and acute but transient depletion of peripheral blood lymphocytes was also observed. Compared with the nonhuman primate model, the ferret model offers a readily available and less-expensive alternative with decreased risk for fatal zoonotic diseases, e.g., *Cercopithecine herpesvirus* I (B virus), and less-complex ethical considerations. On the other hand, the lack of available immunological reagents for the ferret may limit its utility for cell-mediated immunity studies.

The ferret model should be useful to evaluate the level of attenuation of potential H5 vaccine strains; in this context, H5N1 vaccine candidates generated by recombinant reassortment techniques were shown to be attenuated for ferrets (17). Since the ferret has been widely used to evaluate the immunogenicity and efficacy of traditional inactivated influenza vaccines, as well as adjuvanted vaccines (28, 29), live attenuated (20, 21), and DNA vaccine preparations (5, 46), the ferret may also be a useful model for evaluating H5 vaccines. The use of the ferret for the evaluation of efficacy of antiviral drugs may further expand the utility of this model (43). In conclusion, we have demonstrated that two avian H5N1 viruses are highly virulent in ferrets, causing severe viral pneumonia and extensive morbidity. Both viruses spread to organs outside of the respiratory tract, in a way similar to the spread of highly pathogenic avian viruses in chickens. The ferret model may be useful for additional studies on the pathogenicity, immunity, and prevention and control of H5N1 viruses and for other avian influenza virus subtypes that have the potential to emerge as a pandemic threat for humans.

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REFERENCES

1. **Barber, W. H., and P. A. Small, Jr.** 1978. Local and systemic immunity to influenza infections in ferrets. Infect. Immun. **21:**221–228.
2. **Campbell, D., C. Sweet, and H. Smith.** 1979. Comparisons of virulence of influenza virus recombinants in ferrets in relation to their behavior in man and their genetic constitution. J. Gen. Virol. **44:**37–44.
3. **Claas, E. C. J., A. D. M. E. Osterhaus, R. Van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet **351:**472–477.
4. **De Jong, J. C., E. C. J. Claas, A. D. M. E. Osterhaus, R. G. Webster, and W. L. Lim.** 1997. A pandemic warning? Nature **389:**554.
5. **Donnelly, J. J., A. Friedman, J. B. Ulmer, and M. A. Liu.** 1997. Further protection against antigenic drift of influenza virus in a ferret model by DNA vaccination. Vaccine **15:**865–868.
6. **Dybing, J. K., S. Shultz-Cherry, D. E. Swayne, D. L. Suarez, and M. L. Perdue.** 2000. Distinct pathogenesis of Hong Kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. J. Virol. **74:**1443–1450.
7. **Fenton, R. J., A. Clark, and C. W. Potter.** 1981. Immunity to influenza in ferrets. XIV: comparative immunity following infection or immunization with live or inactivated vaccine. Br. J. Exp. Pathol. **62:**297–307.
8. **Fujimoto, S., M. Kobayashi, O. Uemura, M. Iwasa, T. Ando, T. Katoh, C. Nakamura, N. Maki, H. Togari, and Y. Wada.** 1998. PCR on cerebrospinal fluid to show influenza-associated acute encephalopathy or encephalitis. Lancet **352:**873–875.
9. **Gao, P., S. Watanabe, T. Ito, H. Goto, K. Wells, M. McGregor, A. J. Cooley, and Y. Kawaoka.** 1999. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J. Virol. **73:**3184–3189.
10. **Gubareva, L. V., J. A. McCullers, R. C. Bethell, and R. G. Webster.** 1998. Characterization of influenza A/Hong Kong/156/97 (H5N1) virus in a mouse model and protective effect of zanamavir on H5N1 infection in mice. J. Infect. Dis. **178:**1592–1596.
11. **Haff, R. F., P. W. Schriver, C. G. Engle, and R. C. Stewart.** 1966. Pathogenesis of influenza in ferrets. I. Tissue and blood manifestations of disease. J. Immunol. **96:**659–667.
12. **Hatta, M., P. Gao, P. Halfmann, and Y. Kawaoka.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840–1842.
13. **Hinshaw, V. S., R. G. Webster, B. C. Easterday, and W. J. Bean, Jr.** 1981. Replication of avian influenza A viruses in mammals. Infect. Immun. **34:**354–361.
14. **Jakeman, K. J., H. Smith, and C. Sweet.** 1989. Mechanism of immunity to influenza: maternal and passive neonatal protection following immunization of adult ferrets with a live vaccinia-influenza virus haemagglutinin recombinant but not with recombinants containing other influenza virus proteins. J. Gen. Virol. **70:**1523–1531.
15. **Katz, J. M., X. Lu, T. M. Tumpey, C. B. Smith, M. W. Shaw, and K. Subbarao.** 2000. Molecular correlates of influenza A H5N1 virus pathogenesis in mice. J. Virol. **74:**10807–10810.
16. **Leigh, M. W., R. J. Connor, S. Kelm, L. G. Baum, and J. C. Paulson.** 1995. Receptor specificity of influenza virus influences severity of illness in ferrets. Vaccine **13:**1468–1473.
17. **Li, S., C. Liu, A. Klimov, K. Subbarao, M. L. Perdue, D. Mo, Y. Ji, L. Woods, S. Hietala, and M. Bryant.** 1999. Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. J. Infect. Dis. **179:**1132–1138.
18. **Li, S., M. Orlich, and R. Rott.** 1990. Generation of seal influenza virus variants pathogenic for chickens because of hemagglutinin cleavage site changes. J. Virol. **64:**3297–3303.
19. **Lu, X., T. M. Tumpey, T. Morken, S. R. Zaki, N. J. Cox, and J. M. Katz.** 1999. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) virses isolated from humans. J. Virol. **73:**5903–5911.
20. **Maassab, H. F.** 1969. Biologic and immunologic characteristics of cold-adapted influenza virus. J. Immunol. **102:**728–732.
21. **Maassab, H. F., A. P. Kendal, G. D. Abrams, and A. S. Monto.** 1982. Evaluation of a cold-recombinant influenza virus vaccine in ferrets. J. Infect. Dis. **146:**780–790.
22. **Marois, P., A. Boudreault, E. DiFranco, and V. Pavilanis.** 1971. Response of ferrets and monkeys to intranasal infection with human, equine and avian influenza viruses. Can. J. Comp. Med. **35:**71–76.
23. **Matrosovich, M., N. Zhou, Y. Kawaoka, and R. Webster.** 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J. Virol. **73:**1146–1155.
24. **Murphy, B. R., D. L. Sly, N. T. Hosier, W. T. London, and R. M. Chanock.** 1980. Evaluation of three strains of influenza A virus in humans, and in owl, cebus and squirrel monkeys. Infect. Immun. **28:**688–691.
25. **Murphy, B. R., V. S. Hinshaw, D. L. Sly, W. T. London, N. T. Hosier, F. T. Wood, R. G. Webster, and R. M. Chanock.** 1982. Virulence of avian influenza A viruses for squirrel monkeys. Infect. Immun. **37:**1119–1126.
26. **Nishimura, H., S. Itamura, T. Iwasaki, T. Kurata, and M. Tashiro.** 2000. Characterization of human influenza A (H5N1) virus infection in mice: neuro-, pneumo- and adipotropic infection. J. Gen. Virol. **81:**2503–2510.
27. **Perkins, L. E. L., and D. E. Swayne.** 2001. Pathobiology of A/Chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. Vet. Pathol. **38:**149–164.
28. **Potter, C. W., J. S. Oxford, S. L. Shore, C. McLaren, and C. Stuart-Harris.** 1972. Immunity to influenza in ferrets. I. Response to live and killed virus. Br. J. Exp. Pathol. **53:**153–167.
29. **Potter, C. W., S. L. Shore, C. McLaren, and C. Stuart-Harris.** 1972. Immunity to influenza in ferrets. II. Influence of adjuvants on immunization. Br. J. Exp. Pathol. **53:**168–179.
30. **Reed, L. J., and H. Muench.** 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
31. **Renegar, K. B.** 1992. Influenza virus infections and immunity: a review of human and animal models. Lab. Anim. Sci. **42:**222–232.
32. **Reuman, P. D., S. Keely, and G. M. Schiff.** 1989. Assessment of signs of influenza illness in the ferret model. J. Virol. Methods **24:**27–34.
33. **Rimmelzwaan, G. F., T. Kuiken, G. van Amerongen, T. M. Bestebroer, R. A. M. Fouchier, and A. D. M. E. Osterhaus.** 2001. Pathogenesis of influenza A (H5N1) virus infection in a primate model. J. Virol. **75:**6687–6691.
34. **Rowe, T.** 2000. Biocontainment of highly pathogenic avian influenza viruses, p. 16–34. *In* J. Richmond (ed.), Anthology of biosafety, vol. II. Facility design considerations. American Biological Safety Association, Mundelein, Ill.
35. **Scheiblauer, H., A. P. Kendal, and R. Rott.** 1995. Pathogenicity of influenza A/Seal/Mass/1/80 virus mutants for mammalian species. Arch. Virol. **140:**341–348.
36. **Senne, D. A., B. Panigrapy, Y. Kawaoka, J. E. Pearson, J. Suss, M. Lipkind,**

H. Kida, and R. G. Webster. 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis. **40:**425–437.
37. **Shope, R. E.** 1934. The infection of ferrets with swine influenza virus. J. Exp. Med. **60:**49–61.
38. **Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster.** 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology **252:**331–342.
39. **Sidwell, R. W.** 1999. The mouse model of influenza virus infection, p. 981–987. *In* O. Zak and M. A. Sande (ed.), Handbook of animal models of infection. Academic Press, Inc., New York, N.Y.
40. **Smith, H., and C. Sweet.** 1988. Lessons for human influenza from pathogenicity studies with ferrets. Rev. Infect. Dis. **10:**56–75.
41. **Suarez, D. L., M. L. Perdue, N. J. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne.** 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Hong. J. Virol. **72:** 6678–6688.
42. **Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox.** 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science **279:**393–396.
43. **Sweet, C., R. J. Fenton, and G. E. Price.** 1999. The ferret as an animal model of influenza virus infection, p. 989–998. *In* O. Zak and M. A. Sande (ed.), Handbook of animal models of infection. Academic Press, Inc., New York, N.Y.
44. **Toms, G. L. R. A. Bird, S. M. Kingsman, C. Sweet, and H. Smith.** 1977. The behaviour in ferrets of two closely related clones of influenza virus of *differing* virulence for man. Br. J. Exp. Pathol. **57:**37–48.
45. **Tumpey, T. M., X. Lu, T. Morken, S. R. Zaki, and J. M. Katz.** 2000. Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. J. Virol. **74:**1443–1450.
46. **Webster, R. G., E. F. Fynan, J. C. Santoro, and H. Robinson.** 1994. Protection of ferrets against influenza challenge with a DNA vaccine to the hemagglutinin. Vaccine **12:**1495–1498.
47. **Webster, R. G., V. S. Hinshaw, W. J. Bean, K. L. Van Wyke, J. R. Geraci, D. J. St Aubin, and G. Petursson.** 1981. Characterization of an influenza A virus from seals. Virology **113:**712–724.
48. **Yetter, R. A., W. H. Barber, and P. A. Small.** 1980. Heterotypic immunity to influenza in ferrets. Infect. Immun. **29:**650–653.
49. **Yuen, K. Y., P. K Chan, M. Peiris, D. N. C. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. F. Ho, R. Sung, A. F. B. Cheng, and The H5N1 Study Group.** 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet **351:** 467–471.
50. **Zaki, S. R., P. W. Greer, L. M. Coffield, C. S. Goldsmith, K. B. Nolte, K. Foucar, R. M. Feddersen, R. E. Zumwalt, G. L. Miller, A. S. Khan, P. E. Rollin, T. G. Ksziaek, S. T. Nichols, B. W. Mahy, and C. J. Peters.** 1995. Hantavirus pulmonary syndrome: pathogenesis of an emerging infectious disease. Am. J. Pathol. **146:**552–579.
51. **Zambon, M.** 1998. Laboratory containment for influenza A H5N1 viruses: level 2, level 3, or level 3+? Commun. Dis. Public Health **1:**71–72.